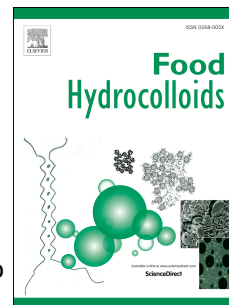


# Accepted Manuscript

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PII: S0268-005X(18)31511-X

DOI: <https://doi.org/10.1016/j.foodhyd.2019.05.043>

Reference: FOOHYD 5142

To appear in: *Food Hydrocolloids*

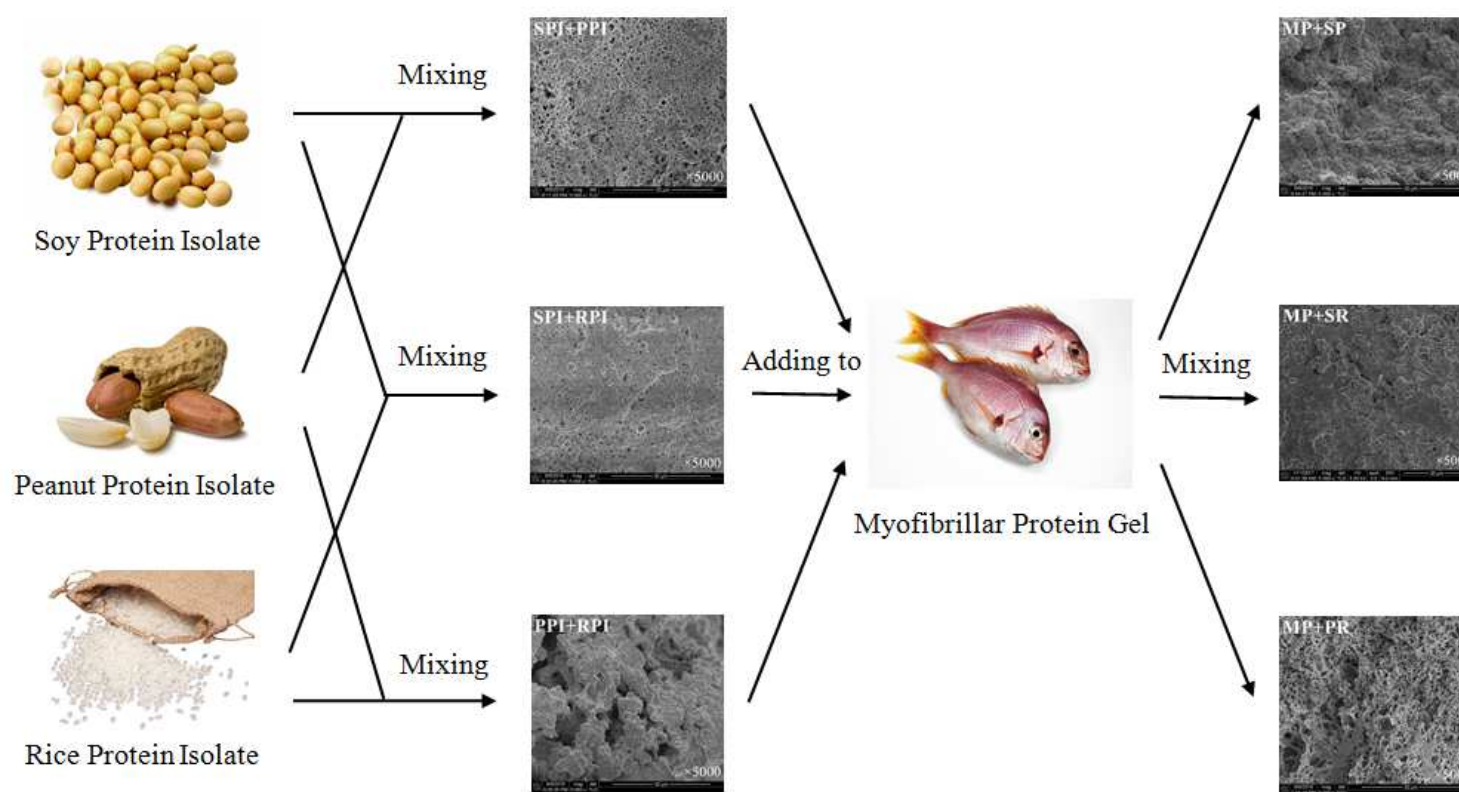
Received Date: 10 August 2018

Revised Date: 9 May 2019

Accepted Date: 24 May 2019

Please cite this article as: Lin, D., Zhang, L., Li, R., Zheng, B., Rea, M.C., Miao, S., Effect of plant protein mixtures on the microstructure and rheological properties of myofibrillar protein gel derived from red sea bream (*Pagrosomus major*), *Food Hydrocolloids* (2019), doi: <https://doi.org/10.1016/j.foodhyd.2019.05.043>.

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**Effect of plant protein mixtures on the microstructure and rheological properties  
of myofibrillar protein gel derived from red sea bream (*Pagrosomus major*)**

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**Abstract**

In this study, the influence of plant protein mixtures (soy protein isolate (SPI) + peanut protein isolate (PPI), SPI + rice protein isolate (RPI), and PPI + RPI) on the microstructure, rheological properties and molecular driving forces of myofibrillar protein (MP) gels was studied. SPI could form a gel with smoother and denser network, while the structures of PPI and RPI gels were rougher, which led to the network structures of SPI + PPI and SPI + RPI gels but the disrupted structure of PPI + RPI gel. However, the SPI + RPI and PPI + RPI gels with different microstructures exhibited larger gel strength compared to the RPI gel. After mixing MP with the mixture of SPI + PPI and SPI + RPI, the mixed gels became more compact, evenner and smoother, while the mixture of PPI + RPI induced more pores to the MP gel. However,  $G'$  values of these three kinds of mixed gels were similar and much larger than that of MP gel. In addition, the molecular driving forces involved in the mixed plant protein gels and mixed MP-plant protein gels were mainly hydrophobic interactions and disulfide bonds.

**Keywords:** Plant protein gel; Myofibrillar protein gel; Rheological property; Microstructure; SEM

## 1. Introduction

Red sea bream (*Pagrosomus major*) is one of the most important species in the aquaculture industry of China, Japan and Korea, due to its great taste, health benefits, and economic feasibility, and the traditional food habits of East Asians (Alam Sarker, Satoh, & Kiron, 2005; Cai et al., 2014). It can be made into traditional snacks, such as fish ball, which is one of the surimi products produced in Fujian Province, China. When surimi is heated to 50 °C, it forms elastic gel, and this process is referred to as 'suwari'. When heated to above 70 °C, a strong gel is formed, and this process is referred to as 'kombako'. The mechanism of surimi formation is that myofibrillar proteins in the fish paste can form a network structure by covalent bonds and non-covalent bonds during heating. However, with the temperature between 50–70 °C the disintegration of the gel structure occurs, and this process is called 'modori'. This is due to strong enzymatic activity of some heat-stable proteases at 60 °C, such as cathepsins, alkaline, proteases, and calpains, which can hydrolyze myofibrillar proteins and ultimately lead to gel weakening (Alvarez, Couso, & Tejada, 1999; An, Peters, & Seymour, 1996).

In addition, people in developed regions consume excessive animal proteins nowadays, which may lead to health problems (Lin, Mouratidou, Vereecken, Kersting, & Bolca, 2015). Animal-based proteins have more saturated fat, cholesterol, and purine compared to plant-based proteins, thus excessive consumption may lead to obesity, coronary heart diseases, high blood pressure, and increased serum and urine uric acid (Bujnowski et al., 2011; Chan, Wang, & Holly, 2007; Tracy et al., 2014).

In order to alleviate problems associated with the protein hydrolyzation during the process of producing surimi products and the health hazards caused by overconsumption of animal proteins, several plant proteins have been incorporated into surimi. Plant proteins cannot only balance the nutritional value of surimi products but also improve their gel strength in two ways. Firstly, they can be used as food-grade protease inhibitors in surimi. For examples, Kudre, Benjakul, and Kishimura (2013) suggested that black bean protein isolate and mung bean protein isolate could partially inhibit proteolysis of sardine modori and kombako gels and increase the breaking force and deformation; however, they decreased whiteness of sardine gels in a concentration-dependent manner. Rawdkuen and Benjakul (2008) also found that whey protein concentrate showed inhibitory activity against proteolysis in surimi, which could increase the strength of some tropical fish gels. Secondly, plant proteins can form a co-gelling or act as binders in surimi gels. In this case, protein-protein interactions including aggregation, phase separation and synergistic interactions occur depending on protein properties and processing technologies (Firoozmand & Rousseau, 2015; Sarbon, Badii, & Howell, 2015). Ramírez-Suárez, Addo, and Xiong (2005) demonstrated that wheat gluten proteins could be used as a texture-enhancing protein to improve the strength of myofibrillar gels treated with microbial transglutaminase.

There are three types of plant proteins: leguminous proteins, oil seeds proteins and cereal proteins. Among them, soy protein isolate (SPI) has been studied widely. SPI has many advantages and functions, such as renewability, biocompatibility,

biodegradability, gel-forming capacity, film-forming capacity, foamability, emulsibility, and processability (Zhang et al., 2016). However, when SPI is used as an alternative material in the food industry, its beany taste and yellowish color can affect the products quality, which may reduce consumers' acceptance of final products (Kim, Varankovich, & Nickerson, 2016). Compared to SPI, peanut protein isolate (PPI) and rice protein isolate (RPI) have lighter colors and fainter scents. It is hypothesized that the use of a mixture of plant proteins as an alternative material may moderate the effect of SPI on the sensory quality as well as present different structural characteristics in the final products. Thus, the aim of this study was to investigate the microstructures and rheological properties of myofibrillar protein gels mixed with different mixtures of plant proteins (SPI + PPI (SP), SPI + RPI (SR), and PPI + RPI (PR)).

## 2. Materials and methods

### 2.1. Materials

Soy, rice and peanut were purchased at a farmer's market (Fuzhou, Fujian, China). Red sea bream (*Pagrosomus major*) was purchased from a seafood market (Xiamen, Fujian, China), and was transported by air under refrigeration to the laboratory. All reagents and solvents were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Preparation of protein isolates from soy, peanut and rice

SPI, PPI, and RPI were prepared according to a method described by Urbonaite, Jongh, Linden and Pouvreau (2015). Soy and peanut were ground into a powder and

defatted with *n*-hexane three times, whereas rice flour did not have to be defatted because of the low fat content but was also pulverized. The defatted soy flour was suspended in deionized water at a ratio of 1:10 (w/w) at 45 °C and stirred for 30 min, while the defatted peanut flour was suspended at a ratio of 1:9 (w/w) at 55 °C and rice flour was suspended at a ratio of 1:8 (w/w) at 45 °C. Then, the pH values of soy flour suspension, peanut flour suspension, and rice flour suspension were adjusted to 8.0, 9.0, and 11.0, respectively, with 5 M NaOH, and all suspensions were stirred for 30 min. The supernatant was collected by centrifugation (30 min, 6,000×*g*, 13 °C) (Allegra X-30R Centrifuge, Beckman Coulter, Inc., Brea, CA USA). Proteins isolates were obtained by isoelectric precipitation (pH 4.5 for SPI and PPI, and pH 5.5 for RPI) by adjusting the pH value with 6 M HCl. After mild stirring of the precipitate for 12 h at 5 °C, the suspension was centrifuged (30 min, 6,000×*g*, 7 °C). The sediment was resuspended three times in deionized water at a ratio of 1:3 (w/w) and filtered by multilayer gauze to remove any remaining insoluble material. The filtrate was centrifuged (30 min, 6,000×*g*, 7 °C) again. The sediment was finally suspended in deionized water at a ratio of 1:4 (w/w), and the pH was justified to 7.0 by using 5 M NaOH. Then, the solution was freeze-dried (FD-1D-80, Beijing Boyikang, Inc., Beijing, China) at -75 °C and 10 Pa for 18 h, and the dried SPI, PPI, and RPI powders were obtained. All samples were kept in polyethylene bags and stored at room temperature. The protein contents of SPI, PPI, and RPI were  $98.89 \pm 0.54$ ,  $97.49 \pm 0.62$  and  $91.20 \pm 1.16$  %, respectively.

### 2.3. Preparation of myofibrillar proteins from red sea bream



Myofibrillar proteins (MP) from red sea bream muscle tissue were prepared according to the method described by Pan and Wu (2014) with a slight modification. The fish was scaled, gutted, and then fish heads were chopped off. Fresh red sea bream muscle tissue was cut into thin sections and washed three times with five volumes of 0.1 M KCl-20 mM Tris-maleate buffer (pH 7.0). Three volumes of KCl-Tris-maleate buffer were added to the muscle tissue, and the specimens were homogenized using a homogenizer (GF-1, Beijing Zhongyi Zhonghe Biotechnology Co., Ltd., Beijing, China). The homogenate was filtered through a nylon mesh (#16) to remove the connective tissues and then centrifuged at  $750\times g$  for 10 min. After centrifugation, the sediment was washed with five volumes of KCl-Tris-maleate buffer and centrifuged again. This procedure was repeated four times. Then, the sediment was mixed with five volumes of cold distilled water, washed by stirring, and centrifuged at  $4,000\times g$  for 10 min. The resulting sediment was further centrifuged at  $12,000\times g$  for 20 min. All the procedures were performed at 5 °C.

## **2.4. Preparation of gels**

### **2.4.1. Preparation of plant protein gels**

Protein solutions with the concentration of 14% (w/w) including SPI, PPI, RPI, SPI + PPI (1:1), SPI + RPI (1:1), and PPI + RPI (1:1) were prepared by mixing in an magnetic stirrer (C-MAG HS 4, IKAMAG<sup>®</sup>, Staufen, Germany) for 2 h at 5 °C. The pH values of all solutions were adjusted to 7.0 by using 0.5 M HCl or 0.5 M NaOH. These solutions were subjected to heat treatment at 90 °C for 30 min, and NaCl was gently added to the hot plant protein solutions by stirring in a magnetic stirrer in order

to obtain the same final ionic strength (FI = 0.5 M) for all solutions. The samples were rapidly cooled to room temperature and incubated at 5 °C for 24 h (Pires Vilela, Cavallieri, & Lopes da Cunha, 2011).

#### 2.4.2. Preparation of mixed myofibrillar protein-plant protein gels

The MP concentration was measured by the Lowry method using bovine serum albumin (BSA) as a reference (Lowry, Rosebrough, Farr, & Randall, 1951). In brief, 1 ml of MP solution after dilution or BSA solutions with concentration from 50 to 250 µg/ml were mixed with 5 ml of reagent (a mixture of 100 ml of reagent A, 2 percent of Na<sub>2</sub>CO<sub>3</sub> and 0.02 percent of seignette salt in 0.10 N NaOH, and 2 ml of reagent B, 0.5 percent of CuSO<sub>4</sub>·5H<sub>2</sub>O in water.), and let them there for 10 min. The mixtures were mixed with 0.5 ml of Folin-Phenol reagent, and let them there for 30 min. The samples were tested in a spectrophotometer at  $\lambda = 500$  nm, and the concentration of MP solution was calculated according to the standard absorbance curve of BSA solutions obtained under the same conditions as the samples.

The concentration of MP was adjusted to 40 mg/mL with 0.5 M NaCl and then different proportions (2%, 6%, 10%, and 14%) of plant protein mixtures (SPI + PPI, SPI + RPI and PPI + RPI) were added to the MP solutions. The solutions were stirred in a magnetic stirrer for 2 h at 5 °C. The obtained solutions were heated in two steps: at 40 °C for 30 min and at 90 °C for 30 min. Finally, the obtained cylindrical complex gels were cooled to room temperature for further analysis, including dynamic rheological measurements, gel solubility, SDS-polyacrylamide gel electrophoresis, and scanning electron microscopy (SEM).

## 2.5. Scanning electron microscopy (SEM)

Microstructures of gels were analyzed using scanning electron microscopy (SEM) according to the method of (Urbonaite, et al., 2015). In brief, gels were sliced (thickness, 2–3 mm) using a razor blade or prepared in 1 mL pipette tips (narrowest part removed, sides cut to aid the diffusion of used reagents) and then immersed in 3% glutaraldehyde solution for 24 h for fixation. After that, samples were washed with deionized water three times for 15 min each time to remove the glutaraldehyde. Before being dried, the samples were protected from freezing by placing them into 50% dimethyl sulfoxide for 24 h. Samples were slowly frozen in liquid nitrogen and then vacuum freeze-dried after peeling the pipette tips away from the gel. Samples were subsequently coated with gold in a sputter coater (Pelco<sup>®</sup>, Ted Pella Inc., Redding, CA, USA) and analyzed in a field emission scanning electron microscope (Nova NanoSEM 230, FEI, Hillsboro, OR, USA).

## 2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to analyze the protein patterns of gels, using a 5% stacking gel (pH 6.8) and a 10% running gel (pH 8.8) (Laemmli, 1970). Insoluble protein gels (2 g) were combined with 10 mL of denaturant solution (100 mM Tris-HCl, pH 8.5) containing 2% SDS (wt/v) and 6 M urea. The mixture was homogenized and then incubated at 85 °C for 30 min. Protein samples were then mixed at a ratio of 3:1 with the SDS-PAGE loading buffer (Beijing solarbio science & technology Co., Ltd., Beijing, China) containing SDS,  $\beta$ -mercaptoethanol, bromophenol blue, and glycerol. The samples

were kept in boiling water for 5 min. Then, the supernatants (5  $\mu$ L) were placed into each well, and a mixture of the marker proteins was deposited in the first lane to identify polypeptides according to their apparent molecular weight (MW). Samples were subjected to electrophoresis at a constant current of 10 mA firstly, and then after samples entered the running gel, the current was changed to 25 mA using a Mini Protean III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Fixed proteins were stained with Coomassie Blue R-250 (0.125%, w/v) in 20% (v/v) ethanol, and destaining was performed by using 5% (v/v) acetic acid and 20% (v/v) ethanol.

## **2.7. Gel solubility**

According to Gómez-Guillén, Borderías, and Montero (1997), five types of solution were used to measure the solubility of the gels to identify molecular driving forces involved in gels: 0.05 mol/L NaCl (SA), 0.6 mol/L NaCl (SB), 0.6 mol/L NaCl + 1.5 mol/L urea (SC), 0.6 mol/L NaCl + 8 mol/L urea (SD), and 0.6 mol/L NaCl + 8 mol/L urea + 0.5 mol/L 2- $\beta$ -mercaptoethanol (SE). Gels (2 g) were mixed with 10 mL of each solution in a magnetic stirrer for 1 h at 5 °C. The resulting homogenates were centrifuged at 20,000 $\times$ g for 15 min using an Allegra X-30R centrifuge. The protein concentration in the supernatants was measured using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The solubility was expressed as g soluble protein/L of homogenate. Each analysis was conducted in triplicate.

## **2.8. Dynamic rheological measurements**

The dynamic rheological analysis was conducted using a rheometer (Anton Paar Physica MCR 301, Graz, Austria) with parallel plate geometry (plate sensor PP-50/P2, 50 mm diameter, 1 mm gap) and a circulating system for temperature control.

The gel samples were loaded on the base plate carefully and equilibrated for 5 min, and then two types of rheological analysis were performed. A strain sweep analysis with an increased from 0.01% to 100% at a frequency of 10 Hz and 25 °C was carried out. Then a frequency sweep analysis was performed between 0.01 and 100 Hz at 25 °C and 0.5% strain to examine the rheological properties of the samples.

## 2.9. Statistical analysis

All measurements were performed on three times and were reported in mean  $\pm$  standard deviation (SD), calculated using SPSS (version 16, IBM, Armonk, NY, USA). Differences between groups were analyzed using analysis of variance and *Tukey's* test, and  $p < 0.05$  was regarded as statistically significant.

## 3. Results and discussion

### 3.1. Microstructure

Microstructures of different plant protein gels are presented in Fig. 1. The plant proteins (SPI, PPI, and RPI) and different combinations of them (SPI + PPI (SP), SPI + RPI (SR), and PPI + RPI (PR)) could form unique three-dimensional network structures; however, all samples had many irregular pores (Hu et al., 2013; Kong, Li, Wang, Hua, & Huang, 2007). Comparing the structures of SPI, PPI and RPI gels, SPI formed a smoother and denser network, while the structures of PPI and RPI gels were rougher. One possible reason for this result was that RPI has very poor solubility

(Shih & Daigle, 2000) and thus the RPI solution is in low viscosity, and RPI molecules is inclined to form an ordered accumulation structure (Clark & Lee-Tuffnell, 1998; Xiong & Brekke, 1989). On the other hand, SPI has better solubility and thus the SPI solution is in higher viscosity, and SPI molecules had stronger association interaction to form a continuous network (Ortiz & Wagner, 2002; Sousa, Morgan, Mitchell, Harding, & Hill, 1996). Moreover, the solubility of PPI and the viscosity of PPI solution seem to be between those of SPI and RPI (Chen, Huang, Wang, Li, & Adhikari, 2016; Hu, Zhao, Sun, Zhao, & Ren, 2011; Malhotra & Coupland, 2004).

In addition, molecular weight of polypeptides can affect the gelation properties of globular proteins and thus the microstructures of protein gels (Totosa, Montejano, Salazar, & Guerrero, 2002). Wang and Damodaran (1990) indicated that the gel strength of globular proteins was related to the size and shape of polypeptides rather than to their chemical nature. The polypeptide compositions of plant proteins gels (SPI, PPI, and RPI) were shown in Fig. 2. SPI mainly contains 7S ( $\beta$ -conglycinin) which is formed by four subunits (i.e.,  $\alpha$ ,  $\alpha'$ ,  $\beta$ , and  $\gamma$ ), and 11S (glycinin) which is formed by acidic and basic subunits. The electrophoretic bands of SPI gel showed the  $\alpha$  (MW 67 kDa),  $\alpha'$  (MW 71 kDa),  $\beta$  (MW 50 kDa), acidic (MW 28–42 kDa), and basic (MW 18–20 kDa) subunits (Lam, Shen, Paulsen, & Corredig, 2007). PPI mainly contains 14S (arachin) which is formed by acidic (MW 37–41 kDa) and basic (MW 19.5 kDa) subunits, 8S ( $\alpha$ -conarachin II, MW 61 kDa), and 2S ( $\alpha$ -conarachin I) which is formed by six subunits (MW 12–17 kDa) (Yang, Chen, & Zhao, 2001). RPI

is mainly composed of gluten, and the electrophoretic bands could be divided into four regions: the HMW group (high-molecular weight subunits of glutenin, MW 80–120 kDa), the MMW group (medium MW  $\alpha$ -gliadins, MW 52–80 kDa), the LMW group (low MW subunits of glutenin,  $\alpha$ - and  $\gamma$ -gliadins, MW 30–52 kDa), and the LMW-NSP (non-storage proteins, MW less than 30 kDa) (Ramírez-Suárez, et al., 2005). As shown in the Fig. 2, the specific electrophoretic bands of PPI and RPI gels showed deep color at the locations of MW 20 kDa or 30 kDa, which may lead to their weaker gelation properties and gel structures than SPI.

Compared with the gel structures of SPI, PPI and RPI gels, the network structures of SPI + PPI gel and SPI + RPI gel had many big pores, while there was not network structure in the PPI + RPI gel as shown in Fig. 1. This indicates that SPI had higher gel-forming ability than PPI and RPI. Thus, SPI molecules could still form a network structure after mixing with PPI or RPI, and the PPI + RPI mixture was inclined to form an accumulation structure. However, after mixing two types of plant proteins, the concentrations of each plant protein decreased to 7 %, which led to decrease in the ability of forming tense network structure. In addition, from the electrophoretic bands of SPI + PPI gel (Fig. 2),  $\beta$ -conglycinin, glycinin, arachin and  $\alpha$ -conarachin II could be identified, respectively, and their color did not change significantly compared to the color of the electrophoretic bands of SPI or PPI gels. The electrophoretic bands of SPI + RPI and PPI + RPI gels showed the same results. No change of color and no formation of new electrophoretic bands indicated that SPI, PPI, and RPI could not react with each other by covalent bonds (except disulfide bonds) after mixing, which

may be another reason for reduction in the ability of forming tense network structures of mixed plant proteins gels.

Fig. 3 shows the microstructures of MP gel and mixed MP-plant proteins gels. The structure of MP gel was uneven and rough with large holes. After mixing MP with the mixture of SPI + PPI and SPI + RPI, the mixed gels became more compact, even and smoother. One possible reason was that, due to the excellent gelation properties of SPI and MP, each protein could form its own structure after mixing, and finally these separate structures interwove together to form an intense structure (Oechsle, Häupler, Gibis, Kohlus, & Weiss, 2015; Sun, Wu, Xu, & Li, 2012). However, the mixture of PPI + RPI induced more pores to the MP gel. This may be because the accumulation structure formed by the mixture of PPI + RPI may occupy the binding sites of MP molecules and weaken its network structure (Lin et al., 2017). These results indicate that the mixtures of SPI + PPI and SPI + RPI could strengthen the microstructure of MP gel, while the mixture of PPI + RPI could disrupt the microstructure of MP gel.

Fig. 4 shows that myofibril protein was comprised of actin (MW 43 kDa), myosin (MW 470 kDa) and troponin, with myosin accounting for about 55–60 %. Myosin had four light chains (MW 20 kDa) and two heavy chains (MW 200 kDa), which closely related to protein-protein interactions (Kudre, et al., 2013). After mixing MP with plant proteins, their specific electrophoretic bands could be respectively identified. However, a lighter color was achieved. This might be explained by the fact that the plant proteins had a certain dilution impact on MP, or adding high level of plant



proteins might hinder the crosslinking of myofibril protein and destroy the network structure of gel. The reason for the destruction is that plant proteins can cross-link to each other after denaturation induced by exposure to extreme environments (e.g., heating above 85 °C, ultrasonic, high pressure, extreme pH or electrical force) and form gels in an appropriate environment (e.g., concentration, pH, ionic strength, temperature, addition of plasticizer and coagulant) (Bainy, Corredig, Poysa, Woodrow, & Tosh, 2010; Denavi et al., 2009). However, MP molecules could interact with each other between 30 and 55 °C to form gels during heating (Sun, Huang, Hu, Xiong, & Zhao, 2014). Thus, it is difficult for plant proteins to react with myofibril proteins to form a uniform and contact structure. However, an intense structure could be formed by controlling the processing conditions or using some special process technologies.

### **3.2. Protein solubility of gels**

The solubility of protein gels in different solvents (e.g., SA, SB, SC, SD, and SE) reflects different molecular reactions (e.g., nonspecific associations, ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide bonds) in the gel structure. The chemical substances in solutions can destroy the corresponding chemical bonds of gels to increase solubility. So the solubility of gels in SA meant nonspecific associations in gels, and the differences of solubility of gels in SA and SB, SB and SC, SC and SD, SD and SE indicated ionic bonds, hydrogen bonds, hydrophobic interactions and disulfide bonds, respectively (Gómez-Guillén, et al., 1997).

Table 1 shows the molecular driving forces involved in mixed plant proteins gels, MP gel, and mixed MP-plant proteins gels. The results show that hydrophobic interactions and disulfide bonds in mixed plant proteins (SPI + PPI, SPI + RPI and PPI + RPI) gels were significantly higher than nonspecific associations, ionic bonds and hydrogen bonds, which indicates that hydrophobic interactions and disulfide bonds played important roles in mixed plant proteins gels. Similar results were found in a wheat gluten gel prepared by the same method described in this paper (Wang et al., 2017). The possible reason was that the heat treatment could influence the process of chemical interactions in gels (Zhang, Xue, Xu, Li, & Xue, 2013). Wang et al. (2017) indicated that with increasing gelation temperatures, ionic and hydrogen bonds were disrupted while hydrophobic interactions and disulfide bonds increased in the wheat gluten gel. However, Babajimopoulos, Damodaran, Rizvi and Kinsella (1983) thought that the contribution of hydrophobic and electrostatic interactions in the soy protein gel were negligible by examining the heat of treatment for formation of soy protein gels with different anions. Thus, it was speculated that processing (e.g. heating and ionic strength) has a significant influence on the chemical interactions in the gel, and the gel formation of plant protein mixtures (SPI + PPI, SPI + RPI and PPI + RPI) with 0.5 M NaCl and the heat treatment at 90 °C was closely associated with hydrophobic interactions and disulfide bonds. Moreover, hydrophobic interactions in the mixed SPI + PPI gel were much higher than those in the mixed SPI + RPI and PPI + RPI gels, while disulfide bonds in the mixed SPI + PPI gel were much lower than those in the mixed SPI + RPI and PPI + RPI gels (Table 1). The possible reason was

that the composition of amino acids of SPI, PPI and RPI are different. Some previous studies indicated that the ratio of hydrophobic amino acids (e.g., isoleucine, valine, leucine, etc.) in soy and peanut proteins was higher than that in rice protein, while the ratio of sulfur containing amino acids (e.g., methionine and cysteine) in rice protein was higher than that in soy and peanut proteins (Hamaker et al., 1992; Shih & Daigle, 2000).

For MP gel as shown in Table 1, nonspecific associations, ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide bonds were all much smaller than those in mixed plant protein gels due to their differences in the protein concentration. However, hydrophobic interactions and disulfide bonds were higher than other molecular driving forces, which was because heating could unfold the superhelix of the tail of myosin molecules, exposing hydrophobic amino acids and sulfhydryl groups to the polar environment and resulting in intermolecular aggregation via hydrophobic forces and disulfide bonds (Gill & Conway, 1989; Ko, Yu, & Hsu, 2007). Similar results were obtained by Zhang, Xue, Xu, Li, and Xue (2013).

After adding different plant protein mixtures (SPI + PPI (SP), SPI + RPI (SR) or PPI + RPI (RP)) to MP gel, hydrophobic interactions and disulfide bonds were still significantly higher than nonspecific associations, ionic bonds and hydrogen bonds as shown in Table 1, which indicated that hydrophobic interactions and disulfide bonds played important roles in mixed MP-plant protein gels. Similar results were obtained in mixed myofibrillar and soy proteins gels (Huang et al., 2010). However, the hydrophobic interactions in MP + 14% SP, MP + 14% SR, MP + 14% PR gels

decreased compared to those in SPI + PPI, SPI + RPI and PPI + RPI gels, which may be because lower water content and denser microstructure decreased the movement of hydrophobic groups (Ma, Cui, Zhao, & Huang, 2002). Besides, the disulfide bonds in MP + 14% SP and MP + 14% SR gels increased maybe due to the network structure introduced by SPI gel and the intense structures of MP-SP and MP-SR gels, while the disulfide bonds in MP + 14% PR gel decreased maybe due to the accumulation structure introduced by the PPI + RPI gel and the disrupted structure of MP-PR gel.

### 3.3. Rheological properties

Fig. 5 shows strain and frequency sweep analysis of single plant protein gels (SPI, PPI and RPI) and mixed plant proteins gels (SPI + PPI, SPI + RPI, and PPI + RPI). These two sweep analysis showed a same result: the strength of plant protein gels ranks in this order: SPI > PPI, SPI + PPI, PPI + RPI > SPI + RPI > RPI. This indicates that combining SPI and PPI or SPI and RPI decreased the gel strength compared to the SPI gel, probably due to the porous network structures of SPI + PPI and SPI + RPI gels (Fig. 1). However combining SPI and RPI or PPI and RPI could improve the gel strength compared to the RPI gel, which indicates that both the network structure introduced by SPI and the accumulation structure introduced by PPI to the SPI + RPI and PPI + RPI gels, respectively, could increase  $G'$  of mixed gels. Oechsle et al. (2015) found that SPI with high molecular weight and good performance formed a mixed interwoven structure with collagen molecules and increased  $G'$  of collagen gel. On the other hand, mixing two kinds of proteins with poor gelation properties may lead to phase separation and disrupted gel structure,

which may decrease the water holding capacity and the water content in gel and thus increase  $G'$  of gel as well (Berland & Launay, 1995; Chen, Kang, & Chen, 2008).

Figs. 6–8 show the effect of different additional levels (2%, 6%, 10%, and 14%) of mixed plant proteins (SPI + PPI, SPI + RPI or PPI + RPI) on the gel strength of mixed gels at 0.5 M and natural pH (6.31–6.58). For the strain sweep analysis (Figs 6A, 7A, and 8A), the critical strain ( $\gamma_0$ ), at which  $G'$  just began to decrease by 5% from its maximum value, was determined according to the method described by Shih, Shih, Kim, Liu and Aksay (1990). The  $\gamma_0$  of all gels were similar, which meant their rupture strength had no significant differences under the same stress. Frequency sweeps (Figs. 6B, 7B, and 8B) reveal that all the  $G'$  of the gel samples were several times larger than their loss moduli ( $G''$ ), suggesting the formation of the gel networks (Almdal, Dyre, Hvidt, & Kramer, 1993).

These two sweep analysis show that when the additional level of mixed plant proteins was 2%, the impacts on the gel strength of mixtures turned out to be insignificant or adverse. According to Sun, Wu, Xu, and Li (2012),  $G'$  of chicken breast salt-soluble protein gels with 2.5% PPI was essentially identical to that of the gels without PPI, which may be because plant proteins were insufficient to form gels under the condition of such low concentrations (Sun & Arntfield, 2012). When the addition percentage of plant protein mixtures increased from 6% to 14%, the gel strength of mixtures increased. This indicates that a higher content of mixed plant proteins had a better effect on increasing the gel strength of MP gel. Firoozmand and Rousseau (2015) explained that a greater proportion of the dissimilar biopolymers

(i.e., proteins) remained in close proximity, producing a more interdigitated network and hence higher  $G'$  values. In addition,  $G'$  values of MP + 14% SP, MP + 14% SR, and MP + 14% PR were similar and much larger than that of MP gel, which indicates that both the network structure and the accumulation structure of gels could increase  $G'$  of mixed gels.

#### 4. Conclusions

This work investigated the microstructures, molecular driving forces, and rheological properties of mixed plant protein gels (SPI + PPI, SPI + RPI and PPI + RPI) and their effect on the structure and properties of MP gel. The results showed that SPI had strong association interaction to form a continuous network, while RPI was inclined to form an ordered accumulation structure. SPI or PPI could enhance the gel strength of RPI gel after being mixed together, even though the microstructures of SPI + RPI and PPI + RPI gels were different. Moreover, higher content of mixed plant proteins showed a better effect on increasing the gel strength of MP gel, and different combinations of plant proteins lead to different microstructures of mixed MP-plant protein gels but with similar gel strength. Compared with nonspecific associations, ionic bonds, and hydrogen bonds, hydrophobic interactions and disulfide bonds were two main forces in the mixed plant protein gels and mixed MP-plant protein gels.

#### Acknowledgement

Financial support for this work was supported by National Natural Science Foundation of China (31628016).

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- 568
- 569

570 **Table 1** Molecular driving forces involved in different protein gels.<sup>a</sup>

	14% SPI + PPI	14% SPI + RPI	14% PPI + RPI	MP	MP + 14% SP	MP + 14% SR	MP + 14% PR
Nonspecific associations (g/L)	5.81±1.37 <sup>a</sup>	5.96±0.50 <sup>b</sup>	3.38±0.14 <sup>a</sup>	0.42±0.05 <sup>a</sup>	7.48±0.33 <sup>a</sup>	5.46±0.14 <sup>a</sup>	3.04±0.23 <sup>a</sup>
Ionic bonds (g/L)	3.43±1.74 <sup>a</sup>	0.85±0.20 <sup>a</sup>	2.16±0.03 <sup>a</sup>	0.19±0.03 <sup>a</sup>	4.86±0.26 <sup>a</sup>	4.12±0.22 <sup>a</sup>	4.08±0.39 <sup>a</sup>
Hydrogen bonds (g/L)	2.86±1.56 <sup>a</sup>	2.05±0.25 <sup>ab</sup>	3.69±0.57 <sup>a</sup>	0.45±0.07 <sup>a</sup>	4.80±0.23 <sup>a</sup>	2.05±0.19 <sup>a</sup>	7.49±0.72 <sup>ab</sup>
Hydrophobic interactions (g/L)	36.40±2.90 <sup>b</sup>	21.42±0.86 <sup>c</sup>	21.19±1.36 <sup>b</sup>	2.45±0.11 <sup>b</sup>	26.91±1.17 <sup>b</sup>	17.77±1.16 <sup>b</sup>	10.37±0.76 <sup>b</sup>
Disulfide bonds (g/L)	23.90±0.80 <sup>c</sup>	46.67±3.48 <sup>d</sup>	55.72±0.96 <sup>c</sup>	4.69±0.36 <sup>c</sup>	32.94±1.87 <sup>c</sup>	61.28±3.02 <sup>c</sup>	29.01±1.60 <sup>c</sup>

571 <sup>a</sup> Values are mean ± standard deviation of three replicates. Different lowercase letters  
572 indicate significant differences among molecular driving forces for each type of  
573 protein gels ( $P < 0.05$ ).

**Figure captions**

**Figure 1** SEM micrographs of SPI (14%, w/w), PPI (14%, w/w), RPI (14%, w/w), SPI + PPI (14%, 1:1), SPI + RPI (14%, 1:1), and PPI + RPI (14%, 1:1) gels with 0.5 M NaCl at pH 7.0.

**Figure 2** SDS-PAGE analyses of plant protein gels.

**Figure 3** SEM micrographs of mixed MP-plant proteins gels with 14% of plant proteins and 0.5 M NaCl at pH range of 6.31–6.65. SP, SR, and PR means the mixtures of SPI and PPI, SPI and RPI, and PPI and RPI, respectively.

Figure 4 SDS-PAGE analyses of mixed MP-plant proteins gels. SP, SR, and PR means the mixtures of SPI and PPI, SPI and RPI, and PPI and RPI, respectively.

Figure 5 (A) Strain and (B) frequency sweep analysis for 14% SPI, PPI, RPI, SPI + PPI (SP, 1:1), SPI + RPI (SR, 1:1), and PPI + RPI (PR, 1:1) gels.

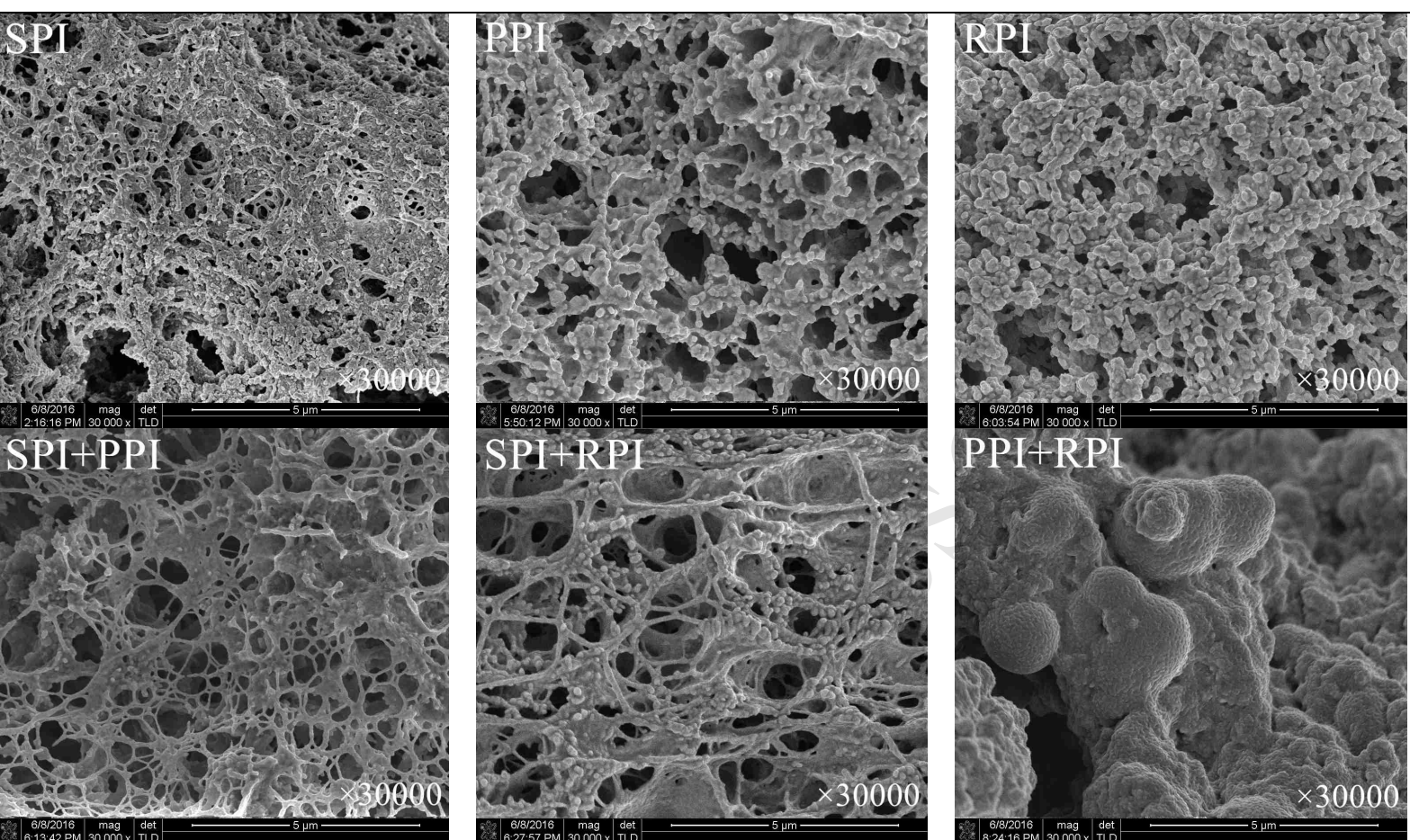
**Figure 6** (A) Strain and (B) frequency sweep analysis for MP, MP + 2% SPI+PPI (SP), MP + 6% SP, MP + 10% SP, and MP + 14% SP gels.

**Figure 7** (A) Strain and (B) frequency sweep analysis for MP, MP + 2% SPI+RPI (SR), MP + 6% SR, MP + 10% SR, and MP + 14% SR gels.

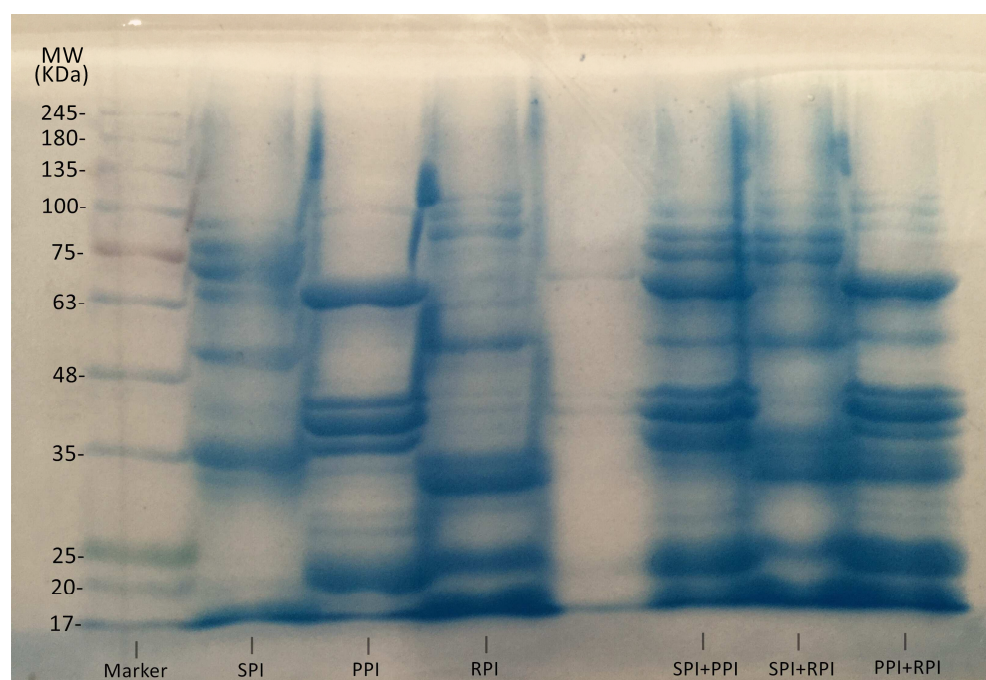
**Figure 8** (A) Strain and (B) frequency sweep analysis for MP, MP + 2% PPI+RPI (PR), MP + 6% PR, MP + 10% PR, and MP + 14% PR gels.

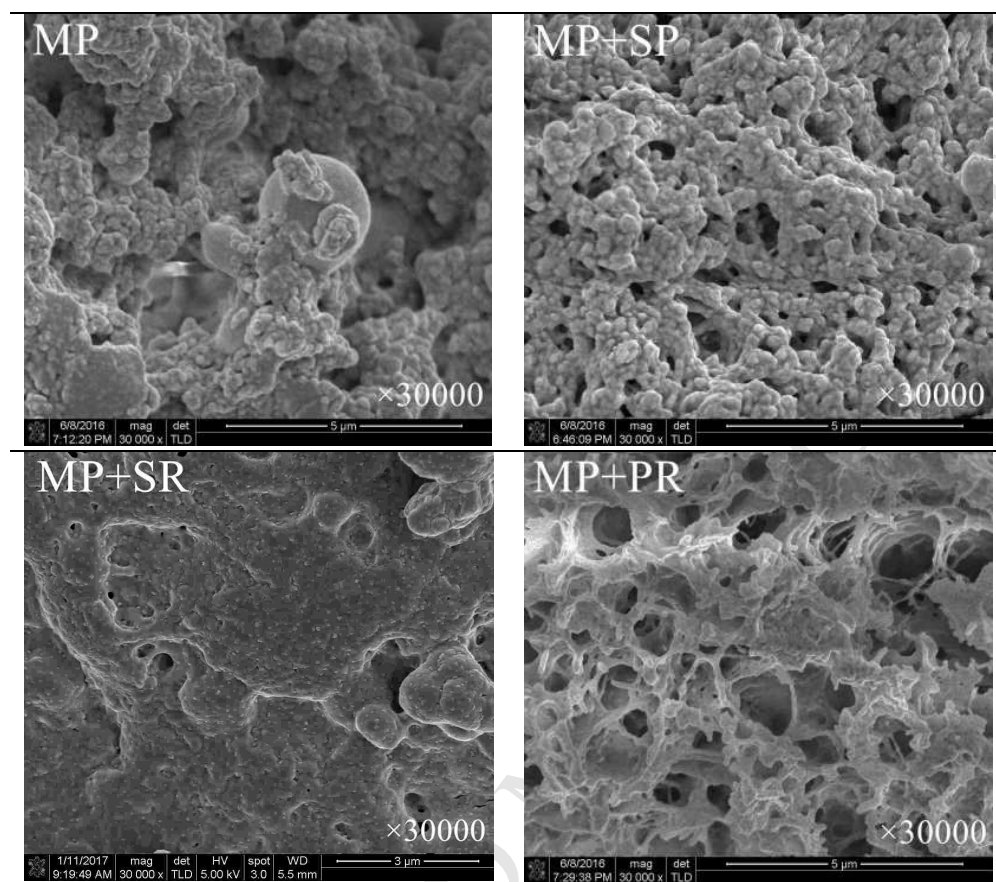


Figure 1





**Figure 2**

**Figure 3**

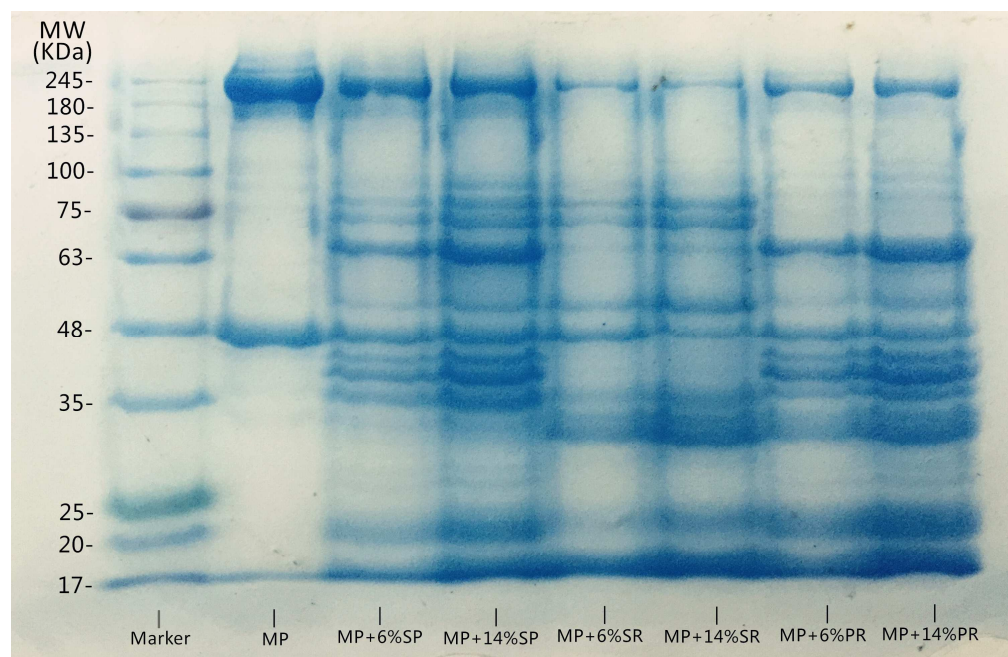
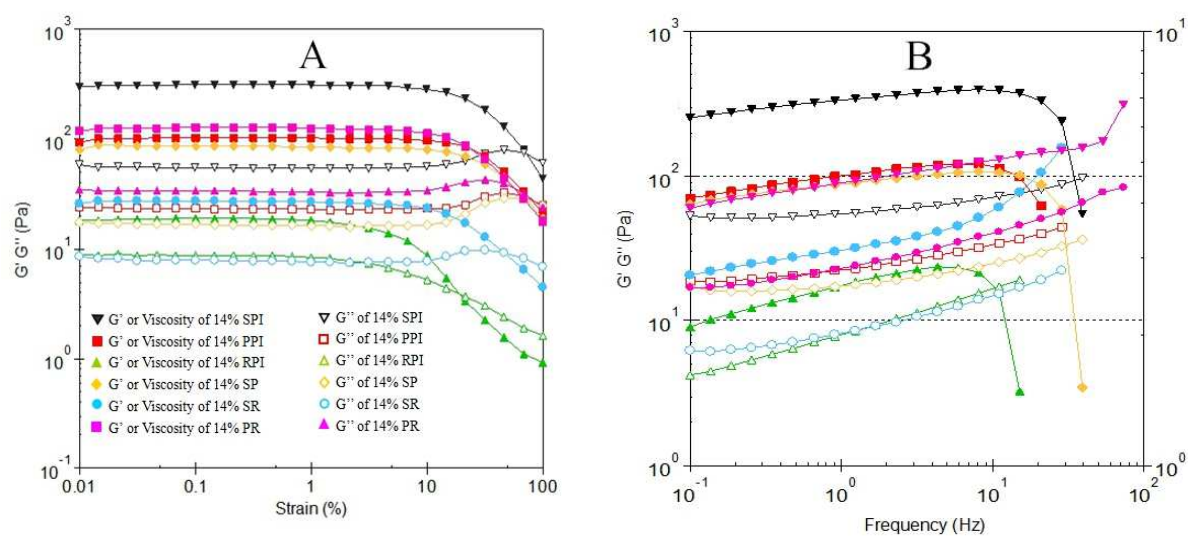
**Figure 4**

Figure 5



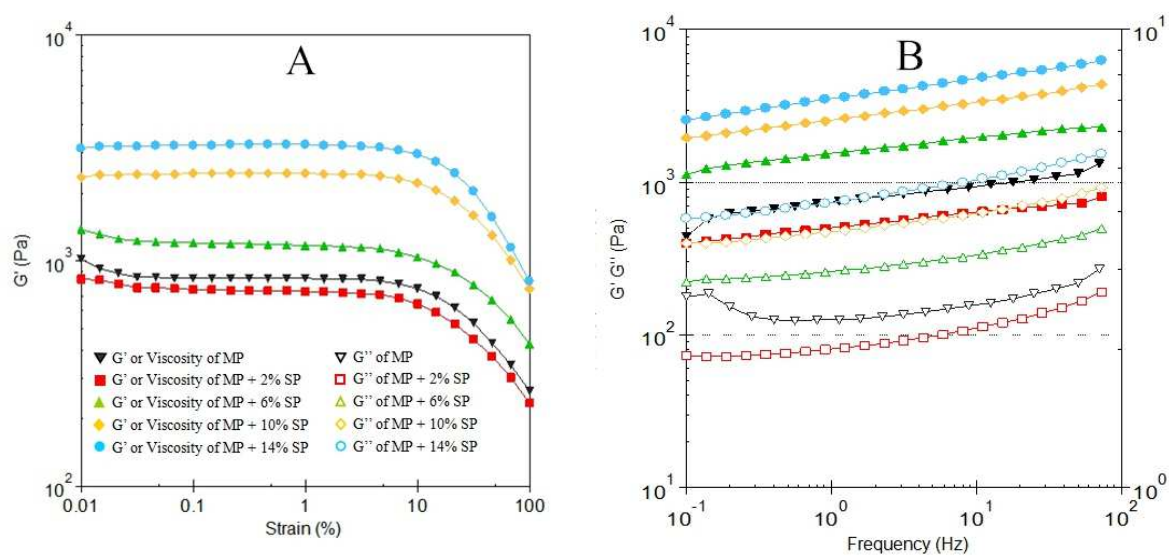
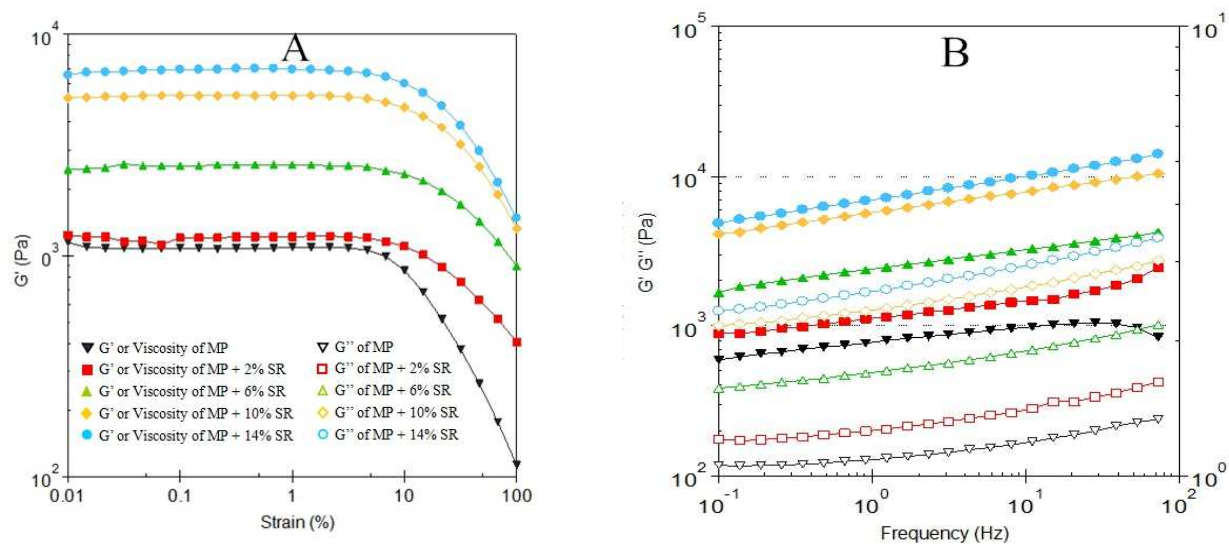
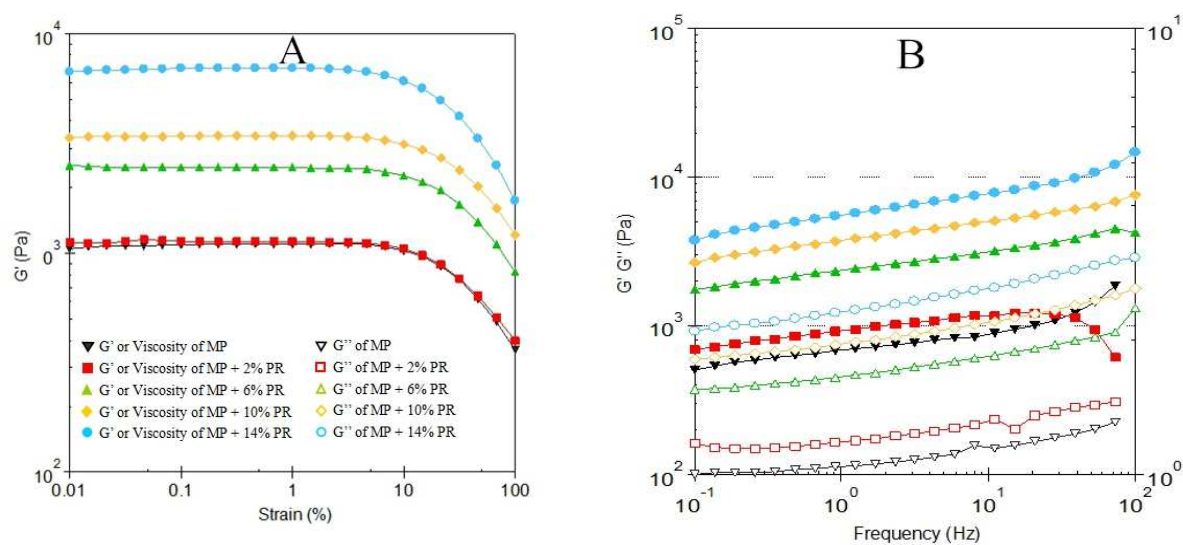
**Figure 6**

Figure 7





**Figure 8**

**Highlights**

- Mixing SPI with PPI or RPI formed gel network while the PPI + RPI gel was disrupted.
- The mixture of SPI + PPI and SPI + RPI made the MP gel more compact and smoother.
- The mixture of PPI + RPI induced more pores to the MP gel.
- Hydrophobic interactions and disulfide bonds were main molecular driving forces in mixed gels.